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Title	: PHARMACEUTICAL COMPOSITION FOR THE	
	: DIAGNOSIS, PREVENTION OR TREATMENT	
	: OF A TUMORAL PATHOLOGY COMPRISING	
	: AN AGENT MODULATING THE	
	: POLYMERIZATION STATE OF ACTIN	
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CLAIM FOR PRIORITY UNDER 35 U.S.C. §119

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Sir:

We submit herewith the certified copy of French Patent Application No. 01/07976, filed June 18, 2001, the priority of which is hereby claimed.

Respectfully submitted,

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VERIFICATION OF TRANSLATION

I, Warwick J Rodden, the below-named translator, hereby declare that:

my name is as stated below;

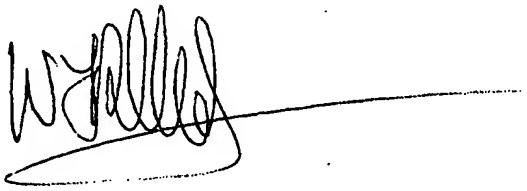
That I am knowledgeable in the English language and in that language in which the below identified Patent Assignment was filed, and that I believe the English translation of Application No. EP 0 821 960.

is a true and complete translation of the above-identified Patent Assignment as filed.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both.

Date: August 2 2006

Full name of Translator: Warwick J. Rodden

Signature of Translator: 

Use of xanthine derivatives for modulation of apoptosis

As opposed to necrosis apoptosis is genetically controlled (programmed) cell death, an essential constituent of the life of multi-cell organisms.

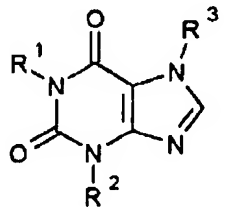
In contrast to this normal and vital apoptosis process countless forms of illness or their symptoms are an expression of an abnormal, i.e. a) sprawling or b) suppressed apoptosis [a): infarction, stroke or neurodegeneration, b) hypertrophic conditions]. Healing procedures of illnesses can thus be possible through suppressing or activating the apoptosis (e.g. paraplegia, immune defence etc.). Following induction of defined death signals, for example via stimulation of certain receptors (e.g. Fas receptor), apoptosis proceeds via a secondary induced complex cascade of interdependent biochemical outcomes, at the end of which is decomposition of the intact cell to membrane-packaged units, which can be disposed of by the body without or with only minor damage to the surrounding cells (as opposed to necrosis). At the same time the transitions between necrosis and apoptosis are in many cases fluent; so there are cases where necrosis results in apoptosis (or vice versa) (e.g. infarction, stroke etc.).

As costimulatory factor in T cells cofilin, a 19-kDa actin-binding protein, plays a decisive role in immune reaction. Cofilin is present in cytosol phosphorylised and is transported into the cell core after dephosphorylising. Here it apparently serves as a carrier molecule for the protein actin, which has no nuclear recognition sequence and is known as DNAse-I inhibitor. Through this mechanism the degree of phosphorylising of

the cytosolic cofilin can have a regulating and modulating influence on the apoptosis of cells.

It has now been found that certain xanthine derivatives are suitable for inhibiting the dephosphorylising of cofilin and they accordingly have a modulating influence on apoptosis.

The invention therefore relates to the use of at least one xanthine derivative of formula I



(I)

and/or where required a stereoisomer form of the xanthine derivative of formula I,
whereby R^2 stands for (C_1-C_4)-alkyl,
one of the radicals R^1 or R^3 stands for a radical of formula II,

$$-(CH_2)_n-R-CH_3 \quad (II)$$

wherein R stands for

a) a covalent single bond and wherein n signifies the whole number zero, 1, 2, 3, 4, 5, 6 or 7,
b) stands for a radical -CO- and wherein n signifies the whole number 1, 2, 3, 4, 5 or 6, or
c) stands for a radical $-C(R^4)(OH)-$ and wherein signifies n the whole number 1, 2, 3, 4, 5 or 6 and
R^4 stands for

a) a hydrogen atom or

b) (C_1-C_3)-alkyl, and

the other radical R^3 or R^1 stands for

a) a hydrogen atom,
b) (C_1-C_7)-alkyl,
c) (C_4-C_8)-cycloalkyl-alkyl or
d) alkyl with 2 to 6 carbon atoms, wherein the carbon chain is interrupted with an oxygen atom,
for manufacturing drugs for modulation of apoptosis.

Xanthine derivatives of formula I are particularly preferably used, whereby
R^2 stands for (C_1-C_4)-alkyl and
one of the radicals R^1 or R^3 stands for a radical of formula II, wherein R stands for

a) a radical -CO- or
b) a radical $-C(R^4)(OH)-$,
and n signifies the whole number 3, 4, 5 or 6 and
R^4 stands for a hydrogen atom or (C_1-C_3)-alkyl and
the other radical R^3 or R^1 stands for (C_1-C_7)-alkyl or (C_4-C_8)-cycloalkyl-alkyl.

Xanthine derivatives of formula I are particularly preferably used, whereby
R^2 stands for (C_1-C_2)-alkyl,
R^1 stands for the radical of formula II, wherein R stands for

a) a radical -CO- or
b) a radical $-C(R^4)(OH)-$,
and n signifies the whole number 3, 4, 5 or 6 and

R^4 stands for a hydrogen atom or (C_1-C_2)-alkyl and
R^3 stands for (C_1-C_7)-alkyl or (C_4-C_8)-cycloalkyl-alkyl.

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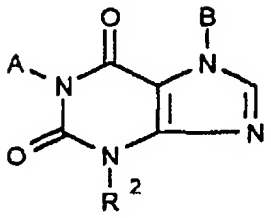
a) a radical -CO- or
b) a radical $-C(R^4)(OH)-$,
and n signifies the whole number 3, 4, 5 or 6 and
R^4 stands for a hydrogen atom or (C_1-C_2)-alkyl and
R^3 stands for (C_2-C_5)-alkyl or (C_4-C_6)-cycloalkyl-alkyl.

1-(5-hydroxy-5-methylhexyl)-3-methyl-7-propyl xanthine is quite particularly preferably used.

The alkyl radicals of formula I are straight-chain or branched. The expression "(C_4-C_8)-cycloalkyl-alkyl" defines such alkyl radicals as are substituted with (C_3-C_6)-cycloalkyl, whereby the sum of all C atoms is smaller than or equal to 8. Examples are cyclopropyl-methyl bis-pentyl-, cyclobutyl-methyl- bis-butyl-, cyclopentyl-methyl- bis-propyl- and cyclohexyl-methyl- and ethyl radical. The radical "(O)" stands for oxygen atom. "Modulation of apoptosis" is understood to mean the inhibiting or induction of apoptosis.

The xanthine derivatives of formula I are produced according to known methods (US 3,737,433; US 4,108,995; US 4,833,146).

One procedure comprises for example that a 3-alkylxanthine of formula II,



(Ia)

in which

R^2 represents an alkyl group with 1 to 4 C atoms,
A stands for a hydrogen atom, (C_4-C_8)-cycloalkyl-alkyl, (C_2-C_6)-alkoxyalkyl or the radical of formula II and
B stands for a hydrogen atom, (C_4-C_8)-cycloalkyl-alkyl, (C_2-C_6)-alkoxyalkyl, the radical of formula II, benzyl- or diphenyl radical, whereby at least one of these radicals A and B signifies a hydrogen atom,
is alkylised directly or in the presence of a basic condensation medium or in the form of one of its salts in 1 or/and 7 position single-stage or progressively with corresponding alkylation signifies of general formula III

X - Q (III)

in which

X signifies a halogen atom or a sulphonic acid ester- or phosphoric acid ester grouping and
Q signifies (C_4-C_8)-cycloalkyl-alkyl, (C_2-C_6)-alkoxyalkyl or radical of formula II,
by subsequent reductive splitting of the radical B, if the latter represents a benzyl or diphenyl methyl group, or where required hydrolytic elimination of an alkoxymethyl radical from the position of the radical B and/or reduction of the keto group for alcohol function,

if A or B signifies an oxoalkyl radical, at a reaction temperature between 0°C and the boiling point of the reaction medium used in each case.

The starting substances of the conversions are known or are easy to produce according to methods known from the literature.

The invention also relates to drugs for modulation of apoptosis, containing at least an effective quantity of a xanthine derivative of formula I, in addition to pharmaceutically suitable and physiologically compatible carriers, dilutants and/or other substances and auxiliaries.

The invention also relates to methods for producing a drug for modulation of apoptosis, which are characterised in that at least one xanthine derivative of formula I with a physiologically acceptable carrier and other suitable substances, additives or auxiliaries are made into an appropriate form.

The inventive drugs are administered parenterally, orally, rectally or where required also topically.

Suitable solid or fluid galenic concoctions are for example granulates, powder, dragees, tablets, (micro)capsules, suppositories, syrups, juices, suspensions, emulsions, drops or injectable solutions, as well as slow-release preparations, during manufacture whereof the usual auxiliaries, such as carriers, explosive, binder, coating, swelling, glide or lubricating agent, flavourings, sweeteners or solubilisers, are used. Frequently used examples of

auxiliaries are e.g. magnesium carbonate, titanium dioxide, lactose, mannite and other sugars, talcum, lactoprotein, gelatine, starch, cellulose and their derivatives, animal and plant oils, polyethylene glycols and solutions, such as sterile water and mono- or multivalent alcohols, e.g. glycerine.

Due to the pharmacological properties of xanthine derivatives of formula I these compounds can be utilised for targeted modulation of apoptosis. Illnesses with spreading apoptosis such as infarction, myoma, muscular atrophy, muscular dystrophy, cachexia, Systemic Inflammation Response Syndrome (SIRS), Adult Respiratory distress Syndrome (ARDS), cerebral malaria, chronic pulmonary inflammation, pulmonary sarcosidosis, reperfusion injuries, keloid, bowel inflammation, Acquired Immune Deficiency Syndrome (AIDS), cancer, illnesses with increased protein loss, stroke, neurodegeneration, chronic renal insufficiency, burn damage or hypertrophic illnesses can be treated.

The pharmaceutical preparations are preferably produced and administered in dosing units, whereby each unit as an active constituent contains a specific dose at least of one xanthine derivative of formula I. In the case of solid dosing forms such as tablets, capsules, dragees or suppositories this dose can be up to approximately 1000 mg, preferably however approximately 100 - 600 mg, and with injection solutions in ampoule form up to 300 mg, preferably 20 - 200 mg. Intravenous infusion treatment of 100 - 2000 mg per day is indicated for treating a patient (70 kg) in early stages. In the later rehabilitation phase oral administration of 3 times 400 mg per day in particular of 1-(5-hydroxy-5-methylhexyl)-3-methyl-7-

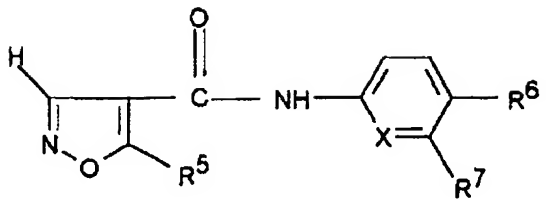
propyl xanthine is indicated. In some circumstances however higher or lower doses are also applied. The dose can be administered both by a one-off dose in the form of an individual dosing unit, or several smaller dosing units, and via multidosing of divided doses at specific intervals.

Finally, xanthine derivatives of formula I and/or where required their corresponding salts can also be formulated during production of the abovementioned galenic preparation forms together with other appropriate substances, for example those which trap free oxygen radicals, e.g. 1,5-hydro-4H-pyrazolo(3,4-d)pyrimidine-4-on, superoxide dismutase, dimethyl sulphoxide or mannitol, heparin, ascorbic acid or deferoxamine.

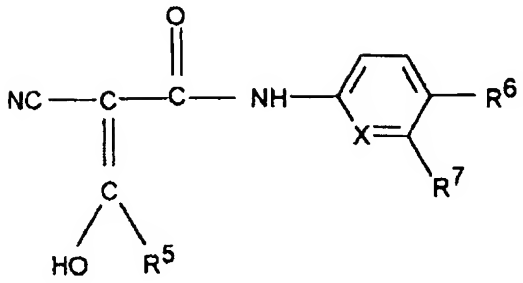
A combination preparation, containing a xanthine derivative of formula I and a compound of formula IV or V, further shows an overadditive inhibiting effect on the dephosphorylising of cofilin and thus on the activating of cofilin, leading to modulation of apoptosis. Due to the extent of this effect the application of this combination preparation can extend to areas for example of immunosuppressive therapy by the individual components closed to now.

The invention therefore further relates to a combination preparation, containing

1) at least one xanthine derivative of formula I as defined hereinabove,
2) a compound of formula IV and/or V,



(IV)



(V)

and/or where required a stereoisomer form of the compound of formula IV or V and/or a physiologically compatible salt of the compound of formula V,
hereby

R^5 stands for

a) $(C_1\text{-}C_4)$-alkyl,
b) $(C_3\text{-}C_5)$-cycloalkyl,
c) $(C_2\text{-}C_6)$-alkenyl or
d) $(C_2\text{-}C_6)$-alkinyl,

R^6 stands for

a) $-CF_3$,
b) $-O\text{-}CF_3$,
c) $-S\text{-}CF_3$,
d) $-OH$,
e) $-NO_2$,

f) halogen,
g) benzyl,
h) phenyl,
i) -O-phenyl,
k) -CN or
l) -O-phenyl, substituted once or more with

1) (C_1-C_4)-alkyl,
2) halogen,
3) $-O-CF_3$ or
4) $-O-CH_3$,

R^7 stands for

a) (C_1-C_4)-alkyl,
b) halogen, or
c) a hydrogen atom, and

X stands for

a) a -CH- group or
b) a nitrogen atom, and

3) a pharmaceutical carrier
for modulation of apoptosis.

The use of a compound of formula IV is preferred, and/or V and/or where required a stereoisomer form of the compound of formula IV or V and/or a salt of the compound of formula V, whereby

R^5 stands for

a) methyl,

b) cyclopropyl or
c) (C_3-C_5)-alkinyl,

R^6 stands for $-CF_3$ or -CN,
R^7 stands for a hydrogen atom or methyl, and
X stands for a -CH- group, in combination with xanthine derivatives of formula I, whereby
R^2 stands for (C_1-C_2)-alkyl,
R^1 stands for a radical of formula II, wherein R stands for

a) a radical -CO- or
b) a radical $-C(R^4)(OH)-$,
and n signifies the whole number 3, 4, 5 or 6 and
R^4 stands for a hydrogen atom or (C_1-C_2)-alkyl and

R^3 stands for (C_2-C_5)-alkyl or (C_4-C_6)-cycloalkyl-alkyl.

The use of N-(4-trifluormethylphenyl)-2-cyan-3-hydroxy-crotonic acid amide, 2-cyano-3-cyclopropyl-3-hydroxy-acrylic acid-(4-cyanophenyl)-amid or N-(4-trifluormethylphenyl)-2-cyan-3-hydroxy-hept-2-en-6-in-carbonic acid amide in combination with 1-(5-hydroxy-5-methylhexyl)-3-methyl-7-propyl xanthine is particularly preferred.

The compound of formula IV or V is made according to known methods, such as described in EP 484 223; EP 529 500; US 4 061 767; EP 538 783 or EP 551 230. The starting materials of the chemical conversions are known or can easily be made according to methods known in the literature.

The term alkyl, alkenyl or alkinyl is understood to mean radicals whereof the carbon chain can be straight-chain or branched. Further, the alkenyl- or alkinyl-radicals may also contain a number of double bonds or respectively a number of triple bonds. Cyclic alkyl radicals are for example 3-bis 5-member monocycles such as cyclopropyl, cyclobutyl or cyclopentyl. The term "overadditive" is understood to mean effects greater than the sum of the individual effects.

The inventive combination preparation is suited for example for treating transplantations, autoimmune illnesses, infarction, stroke, inflammation, neurodegeneration, myoma, muscular atrophy, muscular dystrophy, cachexia, Systemic Inflammation Response Syndrome (SIRS), Adult Respiratory distress Syndrome (ARDS), cerebral malaria, chronic pulmonary inflammation, pulmonary sarcosidosis, reperfusion injuries, keloid, bowel inflammation, Acquired Immune Deficiency Syndrome (AIDS), cancer, illnesses with increased protein loss, stroke, neurodegeneration, chronic renal insufficiency or hypertrophic illnesses.

The inventive combination preparation can also comprise compositions or combination packings, in which the constituents are placed adjacently and can therefore be applied to one and the same human or animal body simultaneously, separately or staggered.

The invention also relates to methods for producing a combination preparation for modulation of apoptosis, which are characterised in that at least one xanthine derivative of formula I and one compound of formula IV or V are brought into a suitable form with a physiologically

acceptable carrier and further suitable active ingredients, additives or auxiliaries.

The inventive combination preparation as a dosing unit can be in the form of drug forms such as capsules (including microcapsules in general containing no pharmaceutical carriers), tablets including dragees and pills, or suppositories, whereby when using capsules the capsule material detects the function of the carrier and the contents can be present e.g. as powder, gel, solution emulsion or dispersion. It is particularly advantageous and simple, however, to produce oral or peroral formulations with both active ingredient components 1) and 2), containing the calculated quantities of the active ingredients together with each desired pharmaceutical carrier. Also a corresponding formulation (suppositories) for rectal therapy can be utilised. Likewise, transdermal application is possible in the form of salves or creams, parenteral (intraperitoneal, subcutaneous, intramuscular) injection or oral application of solutions, containing the inventive combinations. Salves, pastes, creams and powders can contain, apart from the active ingredients, the usual carriers, e.g. animal and plant fats, wax, paraffin, starch, tragacanth, cellulose derivative, polyethylene glycols, silicone, silica gel, aluminium hydroxide, talcum, zinc oxide, lactose, bentite, calcium silicate and polyamide powder or mixtures of these materials.

The tablets, pills or granulates can be produced by methods such as press, dip or fluidised bed methods or boiler sugar-coating, and contain carriers and other usual auxiliaries such as gelatine, agarose, starch (e.g. potato, corn or wheat starch), celluloses such as ethyl

cellulose, silixondioxide, magnesium carbonate, various sugars such as lactose and/or calcium phosphate. Sugar-coating usually comprises sugar and/or starch syrup and mostly contains more gelatine, synthetic cellulose esters, gum Arabic, polyvinyl pyrrolidone, pigments, surface-active substances, emollients and similar additives as per the state of the art.

Every usual flow regulators, lubricating or glide agents such as magnesium stearate and release agents can be used to make the preparation forms. The preparations preferably have the form of mantel/core tablets or multiplayer tablets, whereby the active component 2 is located in the mantel or respectively in the core or respectively in a layer, while the active component 1 is in the core, in the mantel or in another layer. The active ingredients can also be in retarded form or absorbed on retarding material or respectively can be included in the retarding material (e.g. cellulose or polystyrol resin basis, e.g. hydroxyethyl cellulose). Delayed release of the active ingredients can also be achieved by the affected layer or respectively the compartment being provided with the usual gastric juice-insoluble coatings.

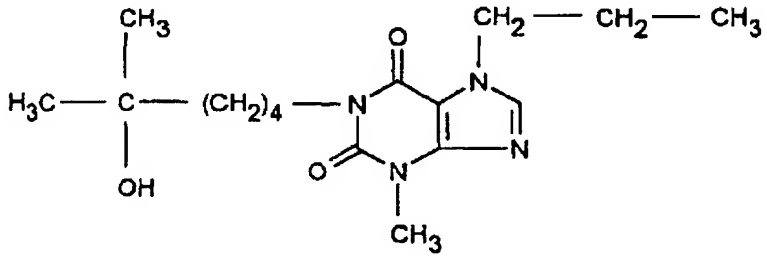
The applicable dosing is naturally dependent on various factors such as the organism to be treated (i.e. human or animal), age, weight, general state of health, degree of severity of symptoms, the illness to be treated, possible companion conditions, (is present) the type of accompanying treatment with other drugs, or frequency of treatment. The dosages are generally administered several times per day and preferably once to three times per day. The used quantities of single active ingredient are

oriented to the recommended daily dosage of the respective single active ingredient and in general should be in the combination preparation from 10 % to 100 % of the recommended daily dosage, preferably from 20 % to 80 %, in particular 50 %.

Appropriate therapy with the inventive combinations thus comprises e.g. administering one, two or 3 single dosages of the preparation comprising N-(4-trifluormethylphenyl-2-cyan-3-hydroxy-crotonic acid amide sodium salt in a quantity of 2 mg to 250 mg, preferably 5 mg to 150 mg, in particular 10 mg to 50 mg, particularly preferably 10 mg to 20 mg and 1-(5-hydroxy-5-methylhexyl)-3-methyl-7-propyl xanthine in a quantity of 100 to 600 mg, in particular from 150 to 300 mg, preferably from 20 to 200 mg.

Example 1

Production of 1-(5-hydroxy-5-methylhexyl)-3-methyl-7-propyl xanthine



22.4 g (0.3 mol) methyl magnesium chloride in the form of 20% solution in tetrahydrofuran is added dropwise to a suspension of 61.3 g (0.2 mol) 3-methyl-1-(5-oxohexyl)-7-propyl xanthine in 2 l water-free ether with strong stirring at room temperature, whereby the inner

temperature rises to approximately 30°C. Next, this is heated for 2 hours with stirring and reflux, mixed with saturated aqueous ammonium chloride solution until the formed alkanolate decomposes; the organic phase is separated off and washed twice with 500 ml water each time. The collected water phases are again extracted basically with dichloromethane. The dichloromethane extract is combined with the etheric phase, dried via sodium sulphate, filtered and concentrated at reduced pressure, whereby 59.0 g raw product (91.5 % in theory) is obtained, which is then cleaned by recrystallising from diisopropyl ether.

Yield: 49.8 g (77.2 % in theory); melting point: 81 - 82°C

$C_{16}H_{26}N_4O_3$ (MG = 322.4)				
Analysis:	Assessed:	59.61 %	H 8.13 %	N 17.38 %
	Found:	59.72 %	H 8.09 %	N 17.44 %

Example 2

Pharmacological Testing

2.1 Cell culture

The murine macrophage cell line RAW 264.7 was coated in ATCC (Rockville, MD) and cultivated in DMEM (Sigma, St. Louis, MO) with 4.5 g glucose/l, 110 mg sodium pyruval/l, 10 % heat-inactivated FCS (Gibco, Grand Island, NY) and penicillin/streptomycin (50 U/ 50 mg/ml).

The macrophages were all transported for 2 - 3 days and one day prior to commencement of the experiment yielded

$2x10^6$ cells in tissue culture flasks (75 cm^2, Falcon, Becton Dickinson GmbH, Heidelberg, Germany). The cells were supplied with fresh medium and the preparations added to the corresponding concentrations. 1-(5-hydroxy-5-methylhexyl)-3-methyl-7-propyl xanthine (compound 1) was dissolved 20 mM in cell medium. Of this, 100 mu l (100 mu M) and 50 mu l (50 mu M) were pipetted to 20 ml medium. N-(4-trifluormethylphenyl-2-cyan-3-hydroxy-crotonic acid amide sodium salt (compound 2) was dissolved 12 mM in cell medium. Of this for every 100 mu l (60 mu M final concentration), 33 mu l (20 mu M final concentration) and 16.7 mu l (10 mu M final concentration) were pipetted to 20 ml medium. Stimulation with lipopolysaccharides (LPS; E. coli, Serotype 0127: B 8 Sigma, St. Louis, MO) in a concentration of 10 ng/ml was conducted for 1 hour following pre-incubation with the preparation. Aliquots of a stock solution of lipopolysaccharides (LPS 1 mg/ml in 10 % dimethyl sulphoxide (DMSO)) were diluted with medium to a concentration of 1 mu g/ml and stored at -20°C. The cells were incubated for 24 hours (h) at 37°C in 10 % CO2.

2.2 Sample preparation

All used chemicals were analytically pure or of electrophoresis quality and were supplied by Millipore Co. (Bedford, MA) or Sigma (St. Louis, MO), if other supply sources were not indicated separately.

2-D electrophoresis (2 DE) was conducted using the Investigator System TM (millipore), and the samples were worked according to the instructions of the manufacturer with slight changes. The adherent murine macrophages were washed three times 60 seconds each standing on ice with

10 ml ice-cold PBS. Then the cells were lysated in 1 ml boiling lysis buffer, comprising 0.3 g SDS, 3.088 g DTT, 0.444 g tris HCl and 0.266 g tris base, in 100 ml. The cell lysate was abraded and heated in a 2 ml sample vessel for 10 minutes (min) in boiling water.

Polynucleotides were split by addition of Benzonase™ (Merck, Darmstadt, Germany) in 30 min at 37°C.

At this point of the sample preparation an aliquot was taken, and the protein content was determined according to the Popov method.

For the 2-DE the proteins of the sample were precipitated by dropwise addition to ice-cold acetone (80 % v/v). The sample was cooled for 20 min on ice and then centrifuged at 240 g 10 min. The dried pellet was taken up in one part lysis buffer and four parts of a sample buffer to a protein content of 5 mg/ml. The sample buffer comprises 59.7 g urea, 4.0 ml NP-40, 1.54 g DTT, 5.5 ml carrier ampholytes (pH 3-10, 2-DE optimised) in 100 ml. Undissolved material was separated out prior to electrophoresis by centrifuging the samples at 16000 x g.

2.3 2 DE Gel electrophoresis

High-resolution two-dimensional gel electrophoresis was conducted according to the O'Farrell method with modifications, as described by Garrels. For this purpose the Millipore Investigator TM 2-D Electrophoresis System (Millipore Co., Bedford, MA) was used.

Isoelectric focussing was carried out in glass capillaries (1 mm in diameter) with a 0.08 mm thick thread, which prevents expanding and tearing of the rod.

The IEF-Gel comprises a 4,1 % T, 2.4 % C polyacrylamide matrix, produced from 30.8 % T, 2.6 % C stock solution, 9.5 M urea, 2.0 % (v/v) NP-40, 10 mM chaps and 2 % (v/v) carrier ampholytes (pH 3-10, 2-DE optimised).

As an anode buffer, 0.01 M H_3PO_4 was used as cathode buffer 0.1 M NaOH. Prior to pre-focussing for forming the pH gradient 15 mu l of a sample-coating buffer, comprising 0.5 M urea, 0.2 % (v/v) NP-40, 0.1 % (v/v) carrier ampholytes and 50 mM DTT, were applied. The voltage maximum of 1500 Volt was reached within 90 minutes at a maximum current of 110 mu A/gel. After pre-focussing 20 mu l of the sample (100 mu g protein) and a further 15 mu l coating buffer were applied.

Isoelectric focussing of proteins took place within 18000 Vh. On completion of electrophoresis the rods were cooled on ice and balanced in a buffer, comprising 0.3 M tris base, 0.075 M tris HCl, 6% SDS, 50 mM DTT and 0.01 % bromphenol blue. The rod gels were transferred directly to the surface of the vertical gel of the second dimension or stored until needed at -20°C. The second dimension was conducted in a SDS gradient gel (10 - 17 %) without collecting gel. The gradient was produced by mixing two gel solutions.

A: 100 ml acryl amide (30.5 % T, 1.64 % C), 73 ml Tris (1.5 M, pH 8.8), 123 ml H_2O, 3 ml SDS (10 %), 150 mu l TEMED and 750 mu l ammonium peroxodisulphate (10 %).

B: 170 ml acryl amide, 73 ml tris, 66.78 g glycerine, 3 ml SDS, 150 mu l TEMED, 750 mu l ammonium peroxodisulphate.

Electrophoresis was conducted overnight at constant temperature in a flow buffer, comprising 25 mM Tris-Base, 192 mM glycine and 0.1 % SDS, until the bromphenol blue front was approximately 1 cm away from the end of the gel. On completion of electrophoresis the proteins in the gel were dyed with silver reagent according to Heukeshoven and Dernick.

Analysis of 2-D gel and creating synthetic images was carried out using the BioImage System (BioImage Systems Co.). The resulting protein pattern was scanned by a Kodak Megaplus Camera Model 1.4 and the data were processed by a HAM station.

2.4 Results

The results of the unstimulated check were set at 100 %. The addition of LPS (10 ng/ml) leads to 50 % dephosphorylising of cofilin. Simultaneous application of LPS (10 ng/ml) and compound 1 (100 mu M) results in 10% dephosphorylising of cofilin. The inhibition of the dephosphorylising is therefore 80 % in comparison to the macrophages treated only with LPS.

Table 1 shows the results. In comparison to 100 mu M final concentration 50 mu M final concentration of compound 1 are ineffective. The compound 2 is likewise without effect up to 20 mu M final concentration, at 60 mu M alone it is effective. If compound 1 and compound 2 are combined in a concentration in which each individual

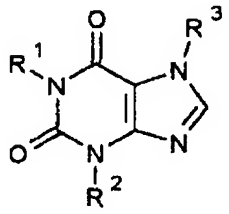
compound is ineffective, the surprising result is an overadditive effect.

Table 1

RAW 264.7 murine macrophages	Intensity of the cofilin spot (%)	Inhibiting of intensity decrease (%)
Check (unstimulated)	100	0
LPS (10 ng/ml)	50	0
LPS + comp. 1 (50 mu M)	50	0
LPS + comp. 1 (100 mu M)	55	10
LPS + comp. 2 (10 mu M)	50	0
LPS + comp. 2 (20 mu M)	50	0
LPS + comp. 1 (50 mu M)+ comp. 2 (10 mu M)	60	20
LPS + comp. 1 (50 mu M)+ comp. 2 (20 mu M)	90	80
comp. stands for compound		

CLAIMS

1. Use of the compound of formula I



(I)

and/or where required a stereoisomer form of the compound of formula I,
whereby R^2 stands for (C_1-C_4)-alkyl,
one of the radicals R_1 or R_3 stands for a radical of formula II,

$$-(CH_2)_n-R-CH_3 \quad (II)$$

wherein R stands for

a) a covalent single bond and wherein n signifies the whole number zero, 1, 2, 3, 4, 5, 6 or 7,
b) stands for a radical -CO- and wherein n signifies the whole number 1, 2, 3, 4, 5 or 6, or
c) stands for a radical $-C(R^4)(OH)-$ and wherein n signifies the whole number 1, 2, 3, 4, 5 or 6 and
R^4 stands for

a) a hydrogen atom or
b) (C_1-C_3)-alkyl, and

the other radical R_3 or R_1 stands for

a) a hydrogen atom,
b) (C_1-C_7)-alkyl,

c) (C_4-C_8)-cycloalkyl-alkyl or
d) alkyl with 2 to 6 carbon atoms, wherein the carbon chain is interrupted with an oxygen atom,
for manufacturing drugs for modulation of apoptosis.

2. Use as claimed in Claim 1, characterised in that a compound of formula I is used, whereby
R^2 stands for (C_1-C_4)-alkyl and
one of the radicals R^1 or R^3 stands for a radical of formula II, wherein R stands for

a) a radical -CO- or
b) a radical $-C(R^4)(OH)-$,
and signifies n the whole number 3, 4, 5 or 6 and
R^4 stands for a hydrogen atom or (C_1-C_3)-alkyl and
the other radical R^3 or R^1 stands for (C_1-C_7)-alkyl or (C_4-C_8)-cycloalkyl-alkyl.

3. Use as claimed in Claim 1 or 2, characterised in that a xanthine derivative of formula I is used, whereby
R^2 stands for (C_1-C_2)-alkyl,
R^1 stands for the radical of formula II, wherein R stands for

a) a radical -CO- or
b) a radical $-C(R^4)(OH)-$,
and n signifies the whole number 3, 4, 5 or 6 and
R^4 stands for a hydrogen atom or (C_1-C_2)-alkyl and
R^3 stands for (C_1-C_7)-alkyl or (C_4-C_8)-cycloalkyl-alkyl.

4. Use as claimed in any one or more of Claims 1 to 3, characterised in that a xanthine derivative of formula I is used, whereby
R^2 stands for (C_1-C_2)-alkyl,

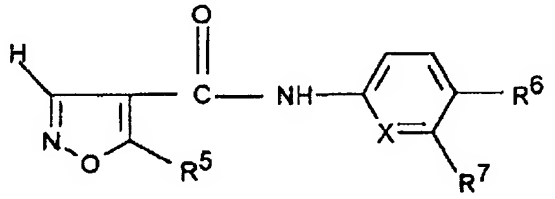
R^1 stands for a radical of formula II, wherein R stands for

a) a radical -CO- or
b) a radical $-C(R^4)(OH)-$,
and n signifies the whole number 3, 4, 5 or 6 and
R^4 stands for a hydrogen atom or (C_1-C_2)-alkyl and
R^3 stands for (C_2-C_5)-alkyl or (C_4-C_6)-cycloalkyl-alkyl.

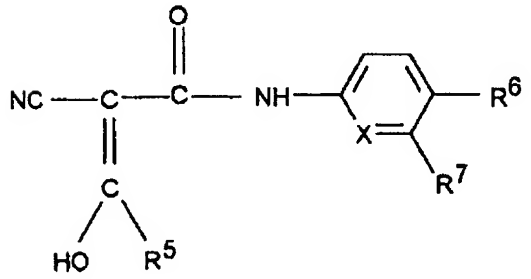
5. Use as claimed in any one or more of Claims 1 to 4, characterised in that 1-(5-hydroxy-5-methylhexyl)-3-methyl-7-propyl xanthine is used.

6. A combination preparation, containing

1) a xanthine derivative of formula I as claimed in Claims 1 to 5,
2) a compound of formula IV and/or V,



(IV)



(V)

and/or where required a stereoisomer form of the compound of formula IV or V and/or a physiologically compatible salt of the compound of formula V,
wereby

R^5 stands for

a) (C_1-C_4)-alkyl,
b) (C_3-C_5)-cycloalkyl,
c) (C_2-C_6)-alkenyl or
d) (C_2-C_6)-alkinyl,

R^6 stands for

a) $-CF_3$,
b) $-O-CF_3$,
c) $-S-CF_3$,
d) -OH,
e) $-NO_2$,
f) halogen,
g) benzyl,
h) phenyl,
i) -O-phenyl,
k) -CN or
l) -O-phenyl, substituted once or more with

1) (C_1-C_4)-alkyl,
2) halogen,
3) $-O-CF_3$ or
4) $-O-CH_3$,

R^7 stands for

a) (C_1-C_4)-alkyl,

b) halogen, or
c) a hydrogen atom, and

X stands for

a) a -CH-group or
b) a nitrogen atom, and

3) a pharmaceutical carrier.

7. The combination preparation as claimed in Claim 6, characterised in that a compound of formula IV and/ or V is used, whereby

R^5 stands for

a) methyl,
b) cyclopropyl or
c) (C_3-C_5)-alkinyl,

R^6 stands for $-CF_3$ or -CN,
R^7 stands for hydrogen atom or methyl, and
X stands for a -CH- group in combination with xanthine derivatives of formula I, whereby
R^2 stands for (C_1-C_2)-alkyl,
R^1 stands for a radical of formula II, wherein R stands for

a) a radical -CO- or
b) a radical $-C(R^4)(OH)-$,
and n signifies the whole number 3, 4, 5 or 6 and
R^4 stands for a hydrogen atom or (C_1-C_2)-alkyl and

R^3 stands for (C_2-C_5)-alkyl or (C_4-C_6)-cycloalkyl-alkyl.

8. The combination preparation as claimed in Claim 6 or 7, characterised in that N-(4-trifluormethylphenyl)-2-cyan-3-hydroxy-crotonic acid amide, 2-cyano-3-cyclopropyl-3-hydroxy-acrylic acid-(4-cyanophenyl)-amid or N-(4-trifluormethylphenyl)-2-cyan-3-hydroxy-hept-2-ene-6-in-carbonic acid amide is used as a compound of formula V and 1-(5-hydroxy-5-methylhexyl)-3-methyl-7-propyl xanthine is used as a xanthine derivative.

9. The combination preparation as claimed in any one of Claims 6 to 8, characterised in that N-(4-trifluormethylphenyl)-2-cyan-3-hydroxycrotonic acid amide and 1-(5-hydroxy-5-methylhexyl)-3-methyl-7-propyl xanthine are used.

10. Use of a compound of formula I as claimed in any one or more of Claims 1 to 5 and a compound of formula IV and/or V according to the definition in one or more of Claims 6 to 9 for producing a drug for modulation of apoptosis.

11. Use as claimed in Claim 10 for treating transplantations, autoimmune conditions, infarction, stroke, inflammations, neurodegeneration, myoma, muscular atrophy, muscular dystrophy, cachexia, Systemic Inflammation Response Syndrome (SIRS), Adult Respiratory Distress Syndrome (ARDS), cerebral malaria, chronic pulmonary inflammation, pulmonary sarcosidosis, reperfusion injuries, keloid, bowel inflammation, burn damage, Acquired Immune Deficiency Syndrome (AIDS), cancer, illnesses with increased protein loss, chronic renal insufficiency or hypertrophic illnesses.

The proteins of the actin cytoskeleton: well placed for motility

m/s2000

Summary

The proteins of the actin cytoskeleton: well placed for motility

The cells of our body are capable of a tremendous variety of movements that require the coordination of the actin cytoskeleton, a complex network of proteins. At the heart of this network, actin is attributed with the dynamic properties of alternating between a monomer and a long polymer chain. One of the major problems in biology has been to understand how the simple process of actin polymerisation and depolymerisation can orchestrate the complex cell movement necessary for embryogenesis, immune response, and wound repair. In this review, we focus on three different, but related systems that play a major role in regulating the actin cytoskeleton. Two of these systems, represented by the Arp2/3 complex and zyxin, appear to be important in controlling actin polymerisation by different mechanisms acting at different locations in the cell. A third major system is represented by ezrin, which, in addition to its direct interaction with actin, can regulate the actin cytoskeleton and affect cell movement and survival. To unravel the complexities of cell movement and the actin cytoskeleton has been a major challenge, but the use of many different experimental systems has now given us an integrated view of cell motility.

On observing living cells, it is possible to recognize the dynamics of the actin cytoskeleton. The cells move in several directions, throw out extensions which are then retracted, can become flattened and at times round–shaped when they divide to give two daughter cells. All these movements require the action of the actin cytoskeleton. Inside a cell this cytoskeleton can be visualised using phalloidin (conjugated with rhodamin), a small molecule which binds to the actin filaments (or actin–F) (*figure 1*). Two types of actin filaments can be seen: actin–F forms a dense network under the plasma membrane, but is also present in the form of highly structured fibres called stress fibres which cross through the cytoplasm and anchor themselves to the plasma membrane in specialised structures called focal adhesion points. The formation of these different structures of actin–F must be coordinated by regulation mechanisms which control cell movement, differentiation, division and survival, hence via events dependent upon the actin cytoskeleton. The polymerisation of actin and the different cell processes it controls undergo extremely fine–tuned regulation in time and space, since the different actin structures are very dynamic in some regions of the cell but not in others. Unlike the complexity of the cytoskeleton observed *in vivo*, the biochemical properties of pure actin in solution are very simple. Monomeric actin (actin–G) polymerises to form filaments, and this polymerisation is controlled by actin concentration in two different manners. Firstly, the kinetics of polymerisation are different at the two ends of the actin filament. One of the ends called the «barbed» or (+) end binds actin monomers at a lower concentration than the other so–called «pointed» or (–) end. This asymmetry imparts dynamics to the actin filaments since it enables one same filament to lengthen at the (+) end and to shorten at the (–) end, at a given actin concentration. The cells use these properties to create new filaments by different methods: either polymerisation occurs using pre–existing filaments through the addition of monomers at the (+) ends (these ends must be uncapped in the cell), or new filament ends are created by the cutting of a filament (fragmentation process). Finally, *de novo* polymerisation of filaments or nucleation from two or three monomers of actin can also occur. Actin polymerisation is also modulated by proteins collectively called «actin binding proteins» which interact with actin–G and/or actin–F. The activity of these proteins is regulated by different signalling pathways, by phosphorylation, by small molecules (Ca^{2+}, phospho–inositides..) or by their concentration at specific sites of the cell. All these processes are responsible for the intense movement seen inside a living cell.

Although the actin cytoskeleton is an entity involved in various cell functions, for reasons of clarity its properties will be discussed herein with respect to its intracellular location. This article will focus on (1) the polymerisation of actin filaments present in the migration front, (2) the organisation of actin filaments in adhesion complexes of moving cells, (3) the interaction of actin filaments with the membrane in dynamic structures of the cell cortex.

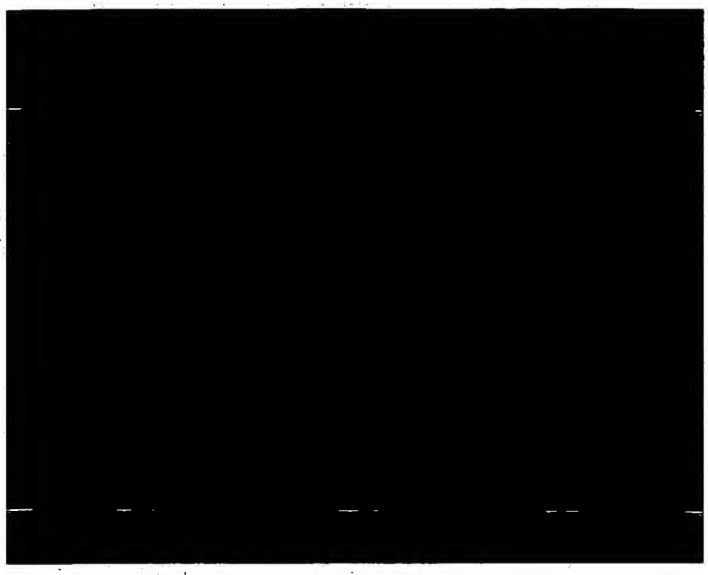


Figure 1. ***Fixed mouse fibroblast labelled with a fluorescent drug binding to actin–F.*** *The most important structures of actin–F can be seen in the migration front (corresponding to the extensions on the right side of the cells) and in the thick bundles of actin–F crossing through the cell. These different structures are produced by different types of proteins, in particular the Arp2/3 complex at the migration front and proteins of the zyxin family found at the focal adhesion points.*

Up front

The Arp2/3 complex

In migration fronts, mobile cells form specialized structures called lamellipodia. This region, approximately 2 µm thick and from which organelles are excluded, contains actin–F which, when observed under electronic microscopy appears to be organized into networks formed of short, branched filaments. In this region of the cell, actin–F incorporates actin monomers quicker than in other regions of the cell to form new filaments. Studies conducted using actin monomers labelled with fluorescent compounds have indicated that new filaments are formed close to the plasma membrane [1]. The polymerisation of actin sets up sufficient forces to cause the plasma membrane to move forward and, with other regions of the cell, to promote cell motility. Previously formed filaments located at the rear of the migration front depolymerise and release actin monomers that are subsequently reincorporated into new filaments close to the plasma membrane. This phenomenon is under strict control and contributes towards producing forces which enable cellular motility.

In 1999, the mechanisms involved in the polymerisation of lamellipodia actin were able to be better understood through converging studies on cell motility reconstitution using bacteria and Xenopus egg extracts [2]. These studies showed that one key element in the production of actin–F is the Arp2/3 complex (*actin related proteins*). This complex consisting of 7 proteins was originally isolated from a unicellular organism, *Acanthamoeba castellanii*, and was later identified in all eukaryote species from yeast to man. The human Arp2/3 complex consists of 7 proteins: Arp2 and Arp3, p41–arc, p34–arc, p21–arc, p20–arc and p16–arc [3]. It is located

in the cell migration front which is the region in which the highest rate of actin polymerisation is observed. Even in yeast, whose cell periphery is limited by a wall, the Arp2/3 complex is associated with cortical structures of actin which are the most dynamic regions.

Initial information on the essential role of the Arp2/3 complex in the polymerisation of actin was obtained by studies conducted with the bacterium *Listeria monocytogenes* [4] (*figure 2*). Listeria are Gram–positive bacteria sometimes found in non–pasteurized dairy products. After ingestion, the bacteria enter into and move within the cells until they reach the plasma membrane and are engulfed by the adjacent cell [5]. The advantage of the Listeria model when examining actin polymerisation was recognized for the first time by Lew Tilney and Dan Portnoy in 1989 [6]. On electronic microscope examination of Listeria–infected macrophages, the authors observed the presence of actin «comets» tailing the bacteria. Actin–F has similar organisation in these comets and in the migration front of moving cells; numerous networked, small filaments can be seen with their (+) ends close to the bacterium (*figure 3*). When Listeria–infected cells are treated with Cytochalasin D toxin, which binds to the (+) end of actin filaments and blocks polymerisation, the bacteria no longer move. In addition, the use of fluorescent actin shows that the actin monomers preferably accumulate at the end of the filaments lying close the bacterium, thereby indicating that the movement of the Listeria is produced by polymerisation of actin at the (+) end, just as in the migration front. The identity of the biochemical mechanisms involved in the movement of Listeria bacteria and in eukaryote cell movement was confirmed by isolating proteins extracted from platelets able to restore bacterial movement. Listeria motility was able to be reconstituted in a system using purified proteins, amongst which the Arp2/3 complex is an essential constituent [7].

Biochemical and kinetic analysis of the interaction of the Arp2/3 complex with actin indicates that the complex binds itself to the (–) end of actin filaments and could stabilize the dimers and trimers of actin required for triggering new polymerisations [8]. This suggests that the Arp2/3 complex has «nucleation» activity i.e. the capacity to assemble actin monomers from which new filaments will be formed (*in vitro* this is a limiting step). However the system has several constraints: it must be activated by another protein, its activity is at its height when it binds to the side of a pre–existing filament, and it remains bound to those filaments for which it induced «nucleation». Although these constraints have led to a re–evaluation of our understanding of the nucleation mechanisms of actin in the migration front, these results tally fully with observations under electronic microscopy. Using a series of spectacular micrographs Svitkina and Borisy have shown that the Arp2/3 complex can be found at the point where new filaments have been formed [9] (*figure 4*). In addition, these branches form an angle of 70° defining an orthogonal network which is similar to those described in the cell migration front and in the Listeria comet.

Despite these highly interesting properties, the Arp2/3 complex only has very weak «nucleation» activity when added to solutions of actin–G, which would suggest that other components are needed. Amongst these, one candidate has been put forward: ActA, a bacterial protein found on the surface of Listeria. The addition of ActA to an actin solution containing the Arp2/3 complex considerably increases the polymerisation rate of actin–G compared with the rate observed without the Arp2/3 complex or ActA. Since eukaryote cells do not usually contain ActA, it has been suggested that another protein cooperating with the Arp2/3 complex must exist in order to stimulate actin polymerisation. The work conducted by Roghatgi *et al* has effectively shown that the proteins of the WASP family also activate the Arp2/3 complex [10].

The identification of WASP and related proteins has made it possible to establish a link between certain diseases and actin polymerisation in the migration front. WASP is the product of the WAS gene which exists in mutated form in patients suffering from Wiskott–Aldrich syndrome. This syndrome, which affects four male children out of every million, is related to

chromosome X and is characterized by severe thrombocytopenia, eczema and immune deficiency (*m/s 1998, n°11, p.1280; m/s 1996, n° 10, p.1173*).

WASP's role in the organisation of actin filaments was initially suggested when its association was evidenced with cdc42, a small GTPase having a regulator role in actin polymerisation [11] (*m/s 1996, n°12, p.1421–3).* Analysis of the primary structure of WASP has revealed that, in addition to the binding site to small GTPases, this protein contains other interaction domains such as a WH1 domain also found among members of the VASP/ena family, a proline–rich domain allowing binding to the SH3 domains. In the carboxy–terminal part, a WH2/VCA domain contains short sequences of amino acids found in verprolin(V) and cofilin(C), two proteins interacting with actin–G and actin–F, and also a sequence required for binding of the Arp2/3 complex.

Like the Arp2/3 complex, the WASP family of proteins is conserved from yeast to man and contains at least three sub–families: VASP/ena, the Scar proteins and WASP. Confirmation of the role played by WASP in producing forces set up by actin polymerisation was provided by an experiment using microbeads coated with the WASP protein. When these beads are placed in a cell extract they recruit actin, form comets and are motile, this movement being dependent upon the Arp2/3 complex [12].

With the identification of the Arp2/3 complex and WASP it finally became possible to explain the polymerisation mechanisms of cell actin. These studies showed that these mechanisms may be more diverse than originally anticipated whilst remaining predictable through mere cell observation.

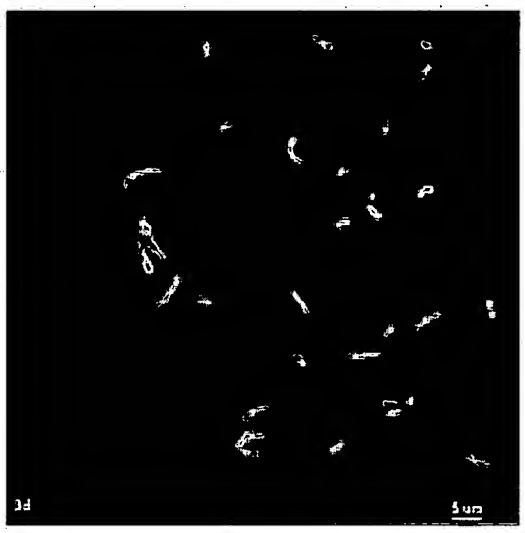


Figure 2. ***Culture cells infected with the bacterium Listeria monocytogenes*** *(in red). On their surface these bacteria express the ActA protein enabling them to recruit proteins from the host cell cytoskeleton. Actin polymerisation by host cell proteins leads to the formation of an actin–F comet (in green) able to promote movement of the bacterium. This type of movement is in fact a simplified version of more complex phenomena observed during cell motility. Molecular analysis of Listeria motility, conducted by different laboratories, was determinant in elucidating the role of the Arp2/3 complex and zyxin for actin polymerisation in human cells (Photo produced by Prof. P. Cossart, Institut Pasteur, Paris, France).*

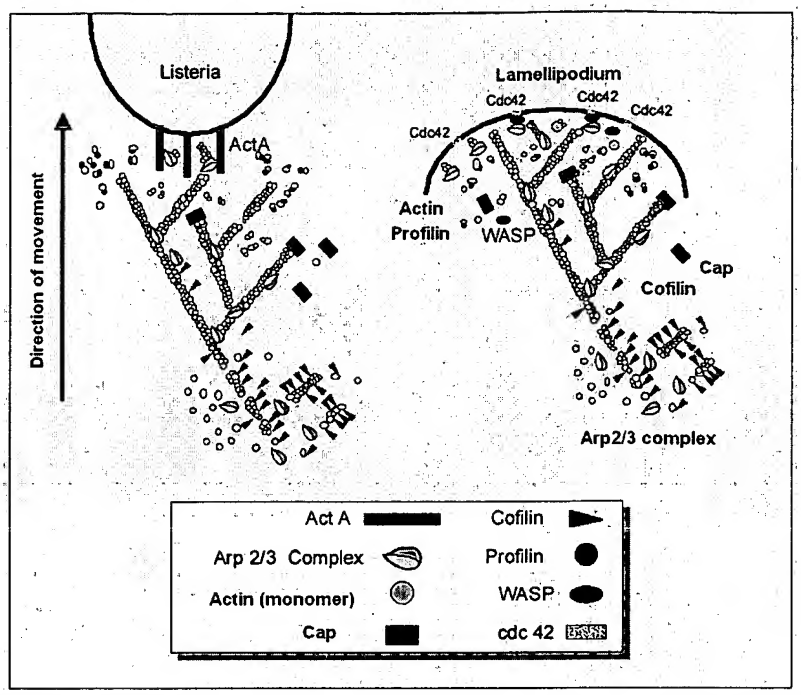


Figure 3. ***Similar organisation of actin in the Listeria comet and in the lamellipodium of a migrating cell.*** *The bacterial protein ActA is responsible for the formation of the actin comet whereas, in the lamellipodium, proteins of the WASP family have very similar activity to protein ActA (Schematic produced by Julie Fradelizi, Institut Curie, Paris, France).*

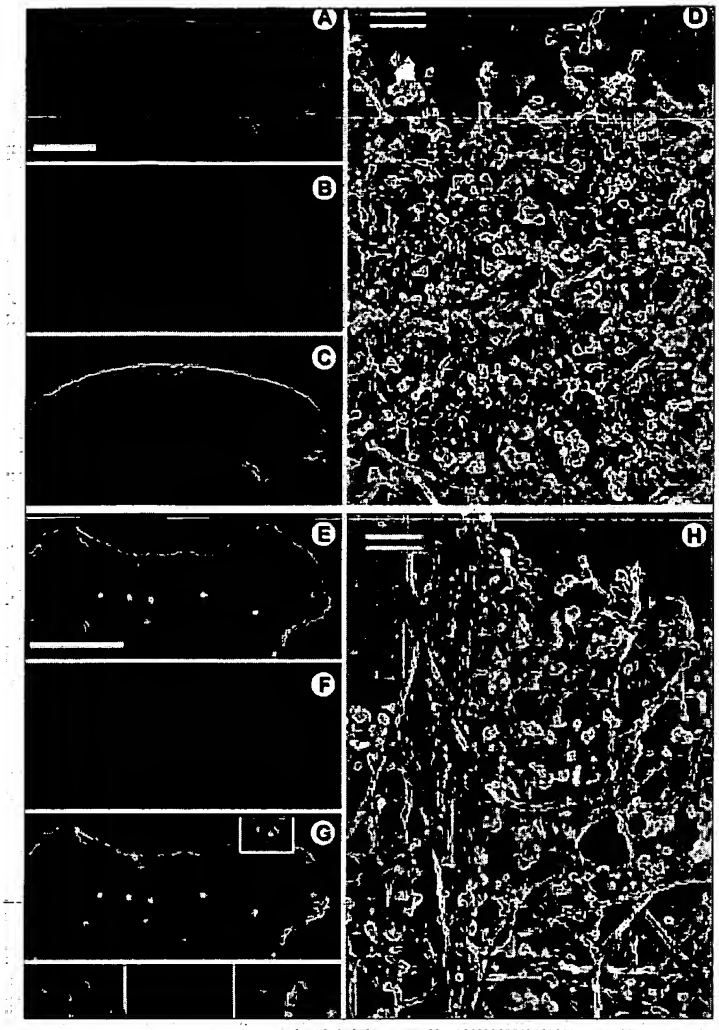


Figure 4. ***Location of the Arp2/3 complex in lamellipodia (a–c and e–g).*** *The cells are keratinocytes (a–c) or Xenopus fibroblasts (e–g). Actin is shown in red and the Arp2/3 complex in green. (**D**) and (**H**) represent visualization under electronic microscopy of Arp 2/3 complexes (yellow) in a keratocyte (**D**) or fibroblast (**H**). (Photo courtesy of G.G. Borisy, web site: http://borisy.bocklabs.wisc.edu.)*

Close behind – zyxin

Aside from the migration front, focal adhesion points represent other structures having a high actin–F content. These anchor points to the extracellular matrix not only have a mechanical function but are also veritable signalling «centres». As in the migration front, it is the dynamic properties of actin associated with the focal adhesions which play an essential role during cell migration. On the other hand, in the focal adhesions, the organisation of actin–F is very different to the organisation seen in the migration front. Unlike in the lamellipodia, the microfilaments are not organized in an orthogonal network but in bundles attached to the plasma membrane. The (+) ends of the microfilaments are oriented towards the plasma membrane and are in close contact with a cytoplasmic protein complex associated with integrins i.e. the transmembrane receptors which anchor the cell to the extracellular matrix. Since the organisation of actin–F in the focal adhesions differs greatly from its organisation in

the migration front, it is perhaps not surprising that these adhesions do not contain the Arp2/3 complex and that another actin polymerisation mode exists in these structures.

It has been well established that the activation of integrins, in tandem with signals derived from tyrosine kinase receptors, triggers the cascade of events leading to the formation of a protein complex anchored to the microfilaments. These signalling pathways activate the small GTPases of the rho family, veritable molecular «switches» which play an essential role in controlling the dynamics and organisation of the cytoskeleton [13]. Therefore one of the functions of Rho is to activate myosin II by controlling the phosphorylation status of the subunit regulating mysonin II [14). In its activated form, myosin II forms bipolar dimers which associate themselves with two actin filaments thereby promoting their organisation into bundles [15]. While the importance of myosin II in the assembly of stress fibres is undeniable, the mechanism through which polymerised actin is formed at the focal adhesions remains ill understood. Study of the assembly of stress fibres under videomicroscopy suggests the existence of a cytoplasmic precursor containing polymerised actin [16]. Similarly the injection of the small rho GTPase into cell cultures induces the formation of stress fibres in the cytoplasm in the absence of adhesion complexes [17]. Bundles of actin could therefore be assembled in the cytoplasm before being anchored to the integrins via proteins such as talin or α–actinin, which interact directly with the cytoplasmic part of integrins. However, experimental data also suggest that actin filaments are formed *de novo* at the focal adhesions. Monomeric actin is rapidly incorporated at these structures in models of permeabilized cells, suggesting that nucleation sites are present in actin polymerisation.

While the Arp2/3 complex is absent from the focal points, it is important to note the presence of zyxin initially discovered by Mary Beckerle at Utah University (USA) [18]. As for the identification of WASP, it is again the Listeria model which confirmed the controlling function of this protein in actin polymerisation. Zyxin is the first mammalian protein for which common structural and functional properties have been evidenced with the ActA of Listeria [19]. Zyxin belongs to a family of proteins located in the lamellipodia, adhesion junctions and focal adhesions. It interacts with the proteins of the cytoskeleton, α–actinin, the VASP/Mena proteins and with Vav an exchange factor of small rac GTPases (*m/s 1995, n°7, p.1045*) and rho GTPases (*m/s 1996, n°11, p.1235–40*). Its carboxy–terminal part, rich in both cystein and histidine residues, comprises three LIM domains. These domains form structures called «zinc fingers» which are involved in protein–protein interactions.

The biochemical and biological properties of zyxin suggest that it is involved in controlling the dynamics of actin. Firstly the targeting of this protein on the inner surface of the plasma membrane via a CAAX sequence induces the formation of actin–rich membrane extensions; secondly, the amino–terminal part of zyxin is proline–rich and comprises «FPPPP» motifs (consensus: E/DFPPPPXD/E). These motifs, initially identified and characterized in the ActA of *Listeria monocytogenes* [20] are also found in LPP a new member of the zyxin family [21] and in vinculin. These properties of zyxin suggest that it could have a role in the assembly of multiprotein complexes taking part in the regulation of actin dynamics. Hence one of the functions of zyxin appears to be that of acting as «anchor point» for proteins of the VASP/Mena family(*figure 5*).

The «FPPPP» motifs are modular units able to bind the members of the VASP/Mena family of which it is thought that they play an essential role in controlling the dynamics of the cytoskeleton. VASP was first described as a substrate of cAMP and cGMP–dependent kinases, activated on inhibition of the aggregating reaction of blood platelets. The Mena protein, another member of this family, is detected solely in the brain [22]. These proteins are related to WASP and, like the latter, contain a EVH1 domain and proline–rich sequences important for the interaction with profilin [23].

Since the detection of the VASP protein on the surface of *Listeria monocytogenes,* its participation in controlling actin polymerisation has been suggested [24]. The motility speed of bacteria producing mutants of ActA, with truncated proline–rich sequences, is reduced three to sixfold. *In vitro* reconstitution experiments of *Listeria monocytogenes* motility have recently confirmed a role of this protein in accelerating the movement of the bacterium [7]. However, contrary to the Arp2/3 complex, the VASP protein is not crucial for bacterium motility, but in its absence speed of movement is reduced by a factor of 10.

The molecular mechanism through which VASP affects the dynamics of the cytoskeleton has not yet been elucidated, since it binds to different components of the actin cytoskeleton. VASP interacts directly with actin–F via its EVH2 domain. Through its binding to profilin, it also interacts with actin–G. It is possible that VASP/Mena, by recruiting the profilin–actin complex, increases the local concentration of actin monomers and hence the rate of polymerisation. Gene inactivation of Mena in mice causes disturbed brain development which is heightened by inactivation of the profilin II–encoding gene [25].

We have seen that at cell level, actin polymerisation is strictly controlled in space and time. This implies that control of space distribution and control of the protein activity required for actin polymerisation are closely linked. Therefore zyxin or other proteins comprising «FPPPP» motifs could be capable of modulating the activity of the VASP/Mena proteins by recruiting these proteins at dynamic sites of the cell. The observation that LPP, related to zyxin, recruits VASP when it is targeted at an ectopic location, points in favour of this type of mechanism [21]. In addition, peptides containing the consensus motif for interaction with VASP/Mena are capable of moving VASP outside the focal adhesions, when added to culture cells [20]. While it is therefore probable that the recruitment of VASP/Mena at adhesion points to the extracellular matrix chiefly depends on proteins comprising «FPPPP» motifs, it is not excluded that VASP/Mena is also directly recruited at sites where actin is incorporated on the inner surface of the plasma membrane [26].

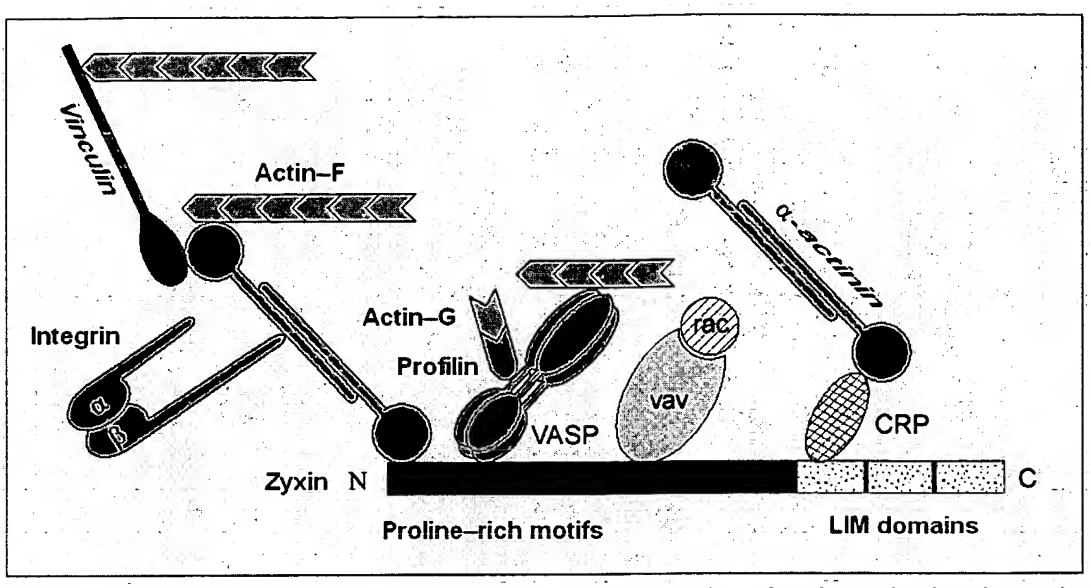


Figure 5. ***Schematic diagram of the structural characteristics of zysin and related proteins.*** *Zyxin contains two domains: a proline–rich domain and a LIM domain. They both contain numerous interaction sites with other proteins of which some interact with the actin cytoskeleton. Here we describe the interaction with VASP, but other partners such as CRP (cystein–rich protein), VAV, α–actinin also interact with zyxin. The complete list of zyxin–binding proteins is not known. Nonetheless it is clear that zyxin and related proteins have a role in regulating the actin cytoskeleton. (Diagram produced by Julie Fradelizi, Institut Curie, Paris, France).*

And overhead – Ezrin

We have described the two types of actin structures visible in the cell as two independent entities but in fact they are not so. When a cell moves and creates a migration front it must set up new contact points. This requires a higher level of organisation similar to the «decision» of a cell to differentiate, divide, continue movement, even to die. All these processes require the actin cytoskeleton and will depend on the coordinated action of polymerisation/ depolymerisation of actin filaments but also on their association with the plasma membrane.
The mode in which the actin filaments interact with the membrane can be most varied but we shall limit this discussion to one family of proteins, the ERMs (ezrin, radixin, moesin). Ezrin was isolated in New York (USA) in 1983 by A. Bretscher at Cornell University (hence its name in tribute to its founder Ezra Cornell) as one of the components of the microvilli of intestinal cells [27], whereas moesin and radixin were characterized later via a molecular biology approach. ERMs have a high degree of homology (75% identity). Also their amino–terminal domain is conserved in a large number of proteins grouped together in a superfamily whose prototype is protein 4.1 [28]. This superfamily also contains merlin/schwannomin, the product of a suppressor gene of tumours responsible for type II neurofibromatosis.
In culture cells, ERMs are present in dynamic, actin–rich structures of the membrane, such as microvilli, filopodia and lamellipodia while in the organs they have specific tissue expression, ezrin and moesin being respectively found at the apical pole of epithelial and endothelial cells [29].
Based on the homology of ERMs with protein 4.1, a binding function of actin filaments with the membrane was suggested for ERMs. However a wait of more than ten years was necessary before initial data could be obtained on the molecular mechanisms involved in the

interaction of ERMs with their partners, and before any functions could be assigned to these proteins.

How do ERMs control the organisation of the cellular cortex? Initial data was obtained by the inactivation of these proteins, or the overexpression of their truncated forms. The MicroCALI technique (chromophore–assisted laser inactivation) has shown that inactivation of ERMs causes retraction of the plasma membrane extensions [30]. The use of antisense oligonucleotides of the mRNAs encoding these three proteins leads to disappearance of the microvilli and changes in the adhesion of the epithelial cells [31]. The overproduction of truncated proteins is often morphogenic and induces membrane extensions [32], whereas these morphogenic effects are not observed with the whole protein.

How can this observation be accounted for? ERMs consist of two domains: a globular amino–terminal domain which interacts with the plasma membrane, and a carboxy–terminal domain associated with actin filaments [33]. The binding site to filamentous actin is located in the last 35 amino acids of the carboxy–terminal domain [34]. Two other potential binding sites to actin–G and F respectively have recently been located in the amino–terminal part of ERMs [35].

The interaction with membrane proteins may be direct: this is the case for the association of ERMs with whole membrane proteins involved in cell adhesion such as CD44, CD43 and ICAMs (intercellular adhesion molecules). On the other hand, the interaction of ERMs with membrane proteins such as CFTR (*cystic fibrosis transmembrane conductance regulator)* or the NA^+/H^+ exchanger is indirect and relayed by phosphoproteins having PDZ domains, the NHE–RF/EBP50 proteins.

The identification of the partners of ERMs has revealed important properties of these proteins. They are able to form intra– and intermolecular interactions via their amino– and carboxy–terminal domains N–ERMAD and C–ERMAD (*ezrin radixin moesin association domains*). These interactions mask the interaction sites of ERMs with their partners. For example, NHE–RF/EBP50 is incapable of binding wild–type ezrin whereas inactivation of the C–ERMAD domain allows this association [36]. The association of these proteins with the membrane and actin cytoskeleton must therefore be regulated by changes in conformation of these proteins, and this theory has been confirmed by various observations. ERMs exist in the cytoplasm in inactive form, and ERM activation leads to oligomerization of these proteins, their recruitment at the membrane and their interaction with actin filaments. However, these different steps have not been formally evidenced and *in vitro* reconstitution experiments should allow analysis of the sequences involved in the activation of ERMs (*figure 6*).

Identification of the signals enabling ERM activation remains a major aspect to be unravelled in order to understand their mode of action. Since ERMs are substrates of various tyrosine and threonine/serine kinases, phosphorylation could be one means of activating these proteins. In several cases, a correlation has been observed between the extent of phosphorylation of ERMs and their association with membrane structures formed in response to stimuli. Conversely, dephosphorylation of the proteins leads to dissociation of ERMS from the plasma membrane [37]. The phosphorylation of a threonine residue located in the binding site to actin–F appears to be directly involved in the interaction of ERMs with actin–F both *in vivo* and *in vitro* [38]. In addition, the phosphorylation of this threonine prevents the interaction of N–ERMAD with C–ERMAD, suggesting that this phosphorylation holds ERMs in an active conformation.

Phosphorylation of ERMs on tyrosine residues is also required for ERM activity. Renal cells cultured in a matrix of type 1 collagen in the presence of HGF (*hepatocyte growth factor*) form structures reminiscent of renal tubules. This tubulogenesis is dependent upon ezrin phosphorylation on tyrosines 145 and 353, since the substitution of these residues by phenylalanines fully inhibits morphogenesis in response to HGF [39]. Finally, phospho–

inositides could play a role in ERM activation since, *in vitro,* PiP2 increases the affinity of ERMs for membrane proteins and actin.

Several observations suggest that there exists a close link between ERMs and the signalling pathway relayed by the small Rho GTPase. In cells transfected with an active form of RhoA (V14RhoA), ERMs phosphorylated on conserved threonine of the carboxy–terminal domain are recruited in RhoA–induced microvilli. The interaction of ERMs *in vitro* with RhoGD1 lifts the Rho inhibition exerted by RhoGD1 and allows its activation through the exchange factor Dbl. Activation of ERMs is required for this interaction [40]. But ERMs also act downstream of Rho activation since they are needed for the formation of Rho–dependent actin fibres and focal adhesions [41]. There appears to exist therefore a regulation loop between the Rho GTPase and ERMs. However activation of ERMs by a Rho effector has not been evidenced.

The work conducted over these last five years has shown that ERMs interact with various membrane partners and different signalling pathways. Little information on the role of ERMs in controlling the organisation of actin filaments has been obtained to date. On the other hand, the involvement of ERMs in various cell functions has already been suggested. For example, ERMs bound to actin filaments could regulate the activity of transmembrane proteins such as CFTR or the Na^+/H^+ exchanger by recruiting regulation proteins [42,43] or such as the β2–adrenergic receptor by regulating its endocytosis [44].

One direct role of ezrin in the transmission of survival signals of epithelial cells has recently been established. Cells producing ezrin whose tyrosine residue 353 has mutated to phenylalanine are no longer able to form tubules in a collagen matrix and enter into apoptosis. The mechanism through which ezrin transmits a survival signal has been identified. The interaction of tyrosine 353 phosphorylated with the C–SH2 domain of P13–kinase leads to activation of P13–K and hence of Akt (*m/s 1999, n° 6/7, p. 897*), a kinase necessary for cell survival. The apoptosis seen in the cells expressing mutated ezrin is hence due to lack of activation of the P13–K/Akt pathway [45]. This experiment therefore provides an example of cell survival regulation via an actin–binding protein.

Ezrin is an example of a protein able to integrate signals which control the dynamics of actin–F and major events such as motility or survival. On account of problems inherent in the study of dynamic systems, it is at times necessary to examine independently the mechanisms of actin polymerisation and the cell processes derived therefrom. Although we have described ezrin, the Arp2/3 complex, zyxin and the systems they represent as isolated entities, these proteins also play an important role in the transmission of signals and the production of actin–F. Complexity is one of the common characteristics of proteins which regulate the actin cytoskeleton and cell motility. The Arp2/3 complex consists of 7 proteins of which 5 have an unknown function, and zyxin and ezrin form multiprotein complexes with an undetermined number of partners. To this structural complexity must be added a space dimension which can be seen in different compartments of a cell. The understanding, at molecular level, of the manner in which the actin cytoskeleton controls cell motility could therefore appear to be an impossible task. However, observation of a developing organism, of tissue formation or of wound repair reveals that these movements are fully controlled. It is perhaps the very elegance of cell motility which prompts us to endeavour to understand its underlying machinery.

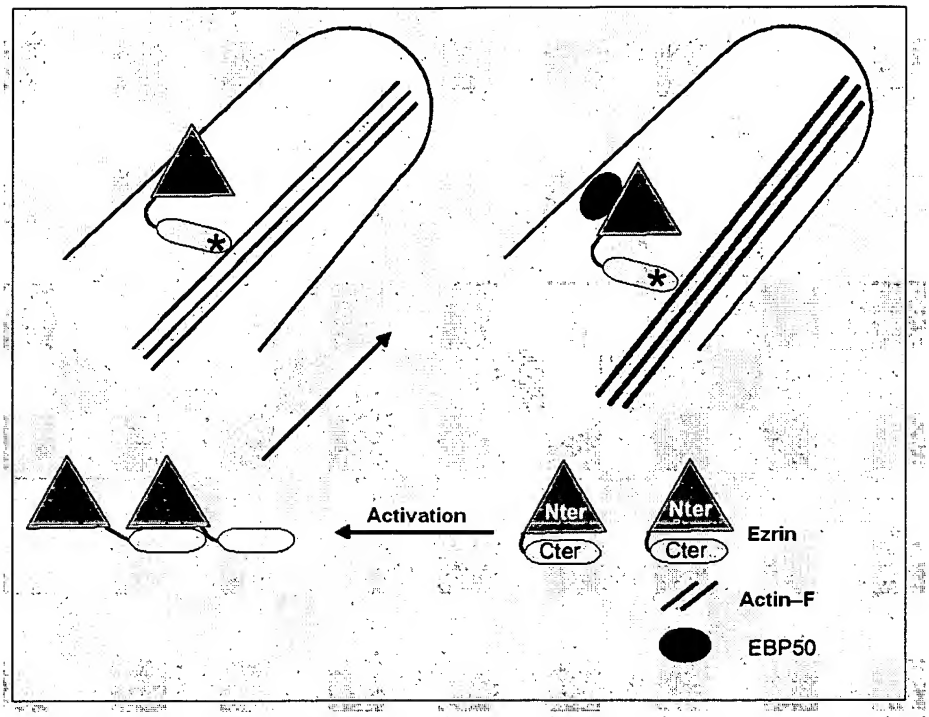


Figure 6. **Model of ezrin activation.** In the cytoplasm, ezrin monomers are in inactive conformation on account of an intramolecular interaction between the amino–terminal domain and the carboxy–terminal domain. Opening the conformation would unmask the cryptic binding sites to membrane proteins and actin filaments. Binding to the membrane proteins may be direct or relayed by a phosphoprotein, NHERF/EBP50. The signals leading to ezrin activation are not known.

ABBREVIATIONS

WASP : Wiskott-Aldrich syndrome protein.
VASP : vasodilator stimulated phosphoprotein.
WH2/VCA : WASP homology 2/verprolin, cofilin, acidic.
WH1 : WASP homology 1.
CFTR : cystic fibrosis transmembrane conductance regulator.
LIM : (Lin-11, Isl-1, Mec-3).
ICAM : intercellular adhesion molecule.